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Presynaptic currents in frog motor endings

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Abstract. Membrane currents were recorded from non-myelinated frog endings by external electrodes. Changes in shape of the signals recorded at varying distances from the myelin end could be explained by assuming a non uniform distribution of Na and K channels along the presynaptic terminal. Specific channel blocking agents revealed that Na channels are present in highest density in the first half of each terminal branch and at almost undetectable levels near the extreme end, while K channels show a more widespread distribution with higher density at medial parts. Suppression of K conductance revealed Ca current which was seen as outward current near the myelin end.

Key words: Motor endings – Ionic channel – Membrane currents

Introduction

We have previously shown (Brigant and Mallart 1982; Mallart and Brigant 1982) in mouse motor endings that Na channels are presents only at the transition between myelinated and unmyelinated segments while K and Ca channels occupy only the remainder of the terminal. We concluded that nerve action potential does not actively invade terminal branches of mouse motor endings which, thus, differ from those of the frog where Katz and Miledi (1965 and 1968) and Braun and Schmidt (1966) demonstrated impulse conduction. This finding was not surprising in view of recent reports on differences in ionic channel distribution between mammalian and amphibian nerves (Chiu et al. 1979; Brismar 1980; Smith and Schauf 1981).

Our previous data were obtained by recording membrane currents by focal external electrodes used in combination with close range ionophoretic application of specific channel blockers. A similar experimental approach is applied here to frog motor terminals to obtain detailed information on channel distribution. It was found that Na, K and Ca channels overlap along most of the length of the terminals, with very low Na channel density at the more distal part.

Methods

Experiments were performed on m. cutaneous pectoris from small green frogs (*Rana esculenta*). Muscles were pretreated with collagenase (Worthington CLSPA, 400 U/ml or Sigma type III, 750 U/ml) to remove as much connective tissue as possible. Standard saline solution contained (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; Hepes, 5 to buffer at pH 7.3. Postsynaptic activity was totally or partially blocked by adding tubocurarine to the continuously perfusing solution. Temperature was maintained at 17°C by means of an electronically controlled Peltier cooling device.

The motor nerve was stimulated by suction electrodes at 1 Hz. Recording electrodes had fire polished tips of 1–2 μm i.d. and were filled with standard saline. Electrodes were positioned on non-myelinated terminals visualized at X500 magnification using a microscope fitted with a Zeiss 40 × immersion lens and Nomarski interference contrast optics. The recordings presented here represent averages of 30–50 sweeps digitized in 20 μs steps by a computer (Intertechnique Didac 800). The recorded signal was the potential drop across the sealing resistance of the electrode, which is proportional to net current traversing the membrane under the electrode. Membrane current is the algebraic sum of inward and outward currents that enter, or leave, the membrane at that point. Ionic current promotes local circuit current which passively traverses the membrane at distant points (Hodgkin and Huxley 1952). Outward current (either ionic or passive) is signaled as upward deflections in the tracings. Amplitude calibrations have been omitted because signal amplitude depended also on factors other than current intensity.

Micropipettes of 200–500 MΩ filled with tetrodotoxin (TTX, 50 μM) or tetraethylammonium (TEA, 0.5 M) and 3,4-diaminopyridine (3,4-DAP, 0.1 M) dissolved in distilled water were used for ionophoretic local drug application. Usually a negative bias of 10 nA was applied to the pipette; drug release was achieved by bias suppression, with effects visible within a few seconds.

Results

Electrical activity along presynaptic terminals

Electric signals recorded along non-myelinated terminal branches are in broad agreement with those described by others in frog muscles (Katz and Miledi 1965; Braun and Schmidt 1966; Benoit and Mambrini 1970; Burley and Jacobs 1981). However, a novel feature was consistently observed in the present experiments: a second negative deflection follows the sharp negative spike in recordings effected very close (0–3 μm) to the myelin end (Fig. 1 A). Relative amplitude of both components varied widely from ending to ending. This

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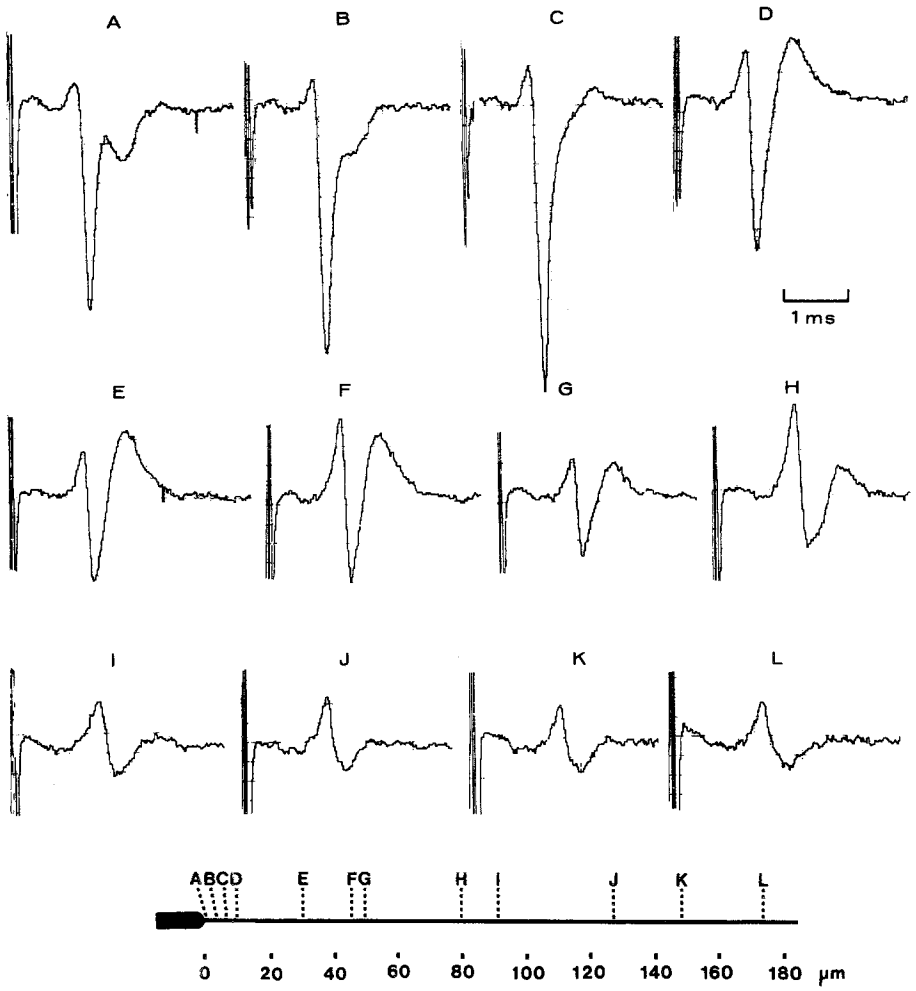

Fig. 1
Membrane currents recorded at electrode positions indicated in the diagram. Distances in μm from the myelin end

waveform closely resembles those recorded in mouse endings at corresponding electrode emplacements (Brigant and Mallart 1982) and in frog myelinated axons (Gundersen et al. 1982). As the electrode was moved in the distal direction, it was possible to record the classical triphasic (positive-negative-positive) and diphasic (positive-negative) waveforms. By careful examination of recordings in Fig. 1, it is possible to follow the evolution of waveform components. The second negative element in A decreases in amplitude in B, starts changing polarity in C and is fully inverted in D and subsequent records. In H, its amplitude declines and seems to start changing polarity again while the negative spike broadens and becomes smaller. This is more evident in I and subsequent records where the negative spike and late positive component have been replaced by a broad negativity (J, K and L). Concomitant changes in other waveform components consist of an enhancement of the early positive deflection and a drastic reduction in size of the negative spike at electrode position beyond the first quarter of the terminal.

Action of TEA and 3,4-DAP on electrical signals

It has been shown that the second positive component of triphasic waveforms corresponds to outward K current associated with propagating action potentials (Benoit and Mambrini 1970; Bostock et al. 1981; Burley and Jacobs 1981). Outward K current can promote a local circuit if areas devoid of K channels exist in the neighborhood, as is the case in mouse motor endings (Brigant and Mallart 1982; Mallart and Brigant 1982). It is thus likely that late negativity of responses recorded at initial and distal portions of frog endings correspond to sinks of current caused by K outflux in medial portions. This hypothesis was tested by applying K channel blockers via bath perfusion or ionophoretic ejection. The effect of bath perfusion of 1 mM TEA + 0.1 mM 3,4-DAP is shown in Fig. 2. Second negative component of proximal responses and negative deflection of diphasic distal signals were readily abolished within a few minutes of drug perfusion. This result may indicate that K channels are only present at medial portions and absent from proximal and distal parts of the endings. Another possibility is that K channels are present all along the terminal but display regional differences in density which determine late current polarity. This view was tested in the experiment shown in Fig. 3. In A, TEA + 3,4-DAP were applied ionophoretically close to the recording electrode positioned at 10 μm from the myelin. Late positivity of the triphasic waveform changed polarity and the signal became similar to that usually recorded close to the myelin end (compare Figs. 2A1 and 3A2). When K channel blockers were applied at electrode positions close to the myelin the second of two negative components increased in amplitude and duration. Similarly, drug release close to recording electrode positioned near the

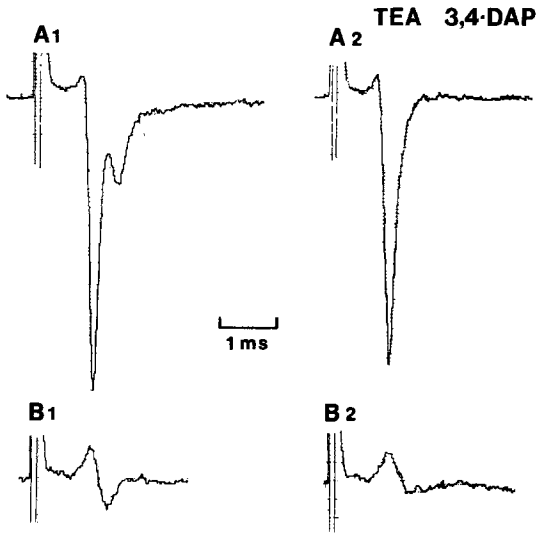

Fig. 2A, B. Action of bath perfusion of K channel blockers. **A1** and **B1** Control responses at proximal and distal parts, respectively, of motor nerve terminal. The effect of bath application of 1 mM TEA + 0.1 mM 3,4-DAP is shown in **A2** and **B2**

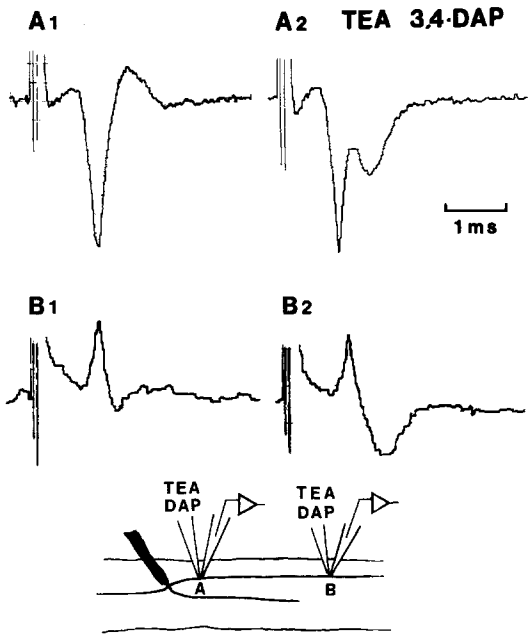

Fig. 3A, B. Ionophoresis of K channel blockers. **A1** Triphasic waveform from the first quarter of the terminal. **A2** Ionophoretic application of TEA + 3,4-DAP close to recording electrode causes reversal of late positive deflection. **B1** Control response from the terminal part. **B2** Local application of both drugs enhances late negativity

distal end induced a large increase in amplitude of the negative component of the diphasic signal (Fig. 3B). These observations indicate the presence of K channels even in areas where net late membrane current is inward.

Side effects of 3,4-DAP such as membrane depolarization (Kirsch and Narahashi 1978) or direct action on Ca channel (Lundh and Thesleff 1980) can be ruled out since Molgó (1982) failed to observe drug dependent changes in miniature end-plate potential frequency in frog end-plates bathed either in normal saline or in a solution containing increased K^+ concentration.

Action of TTX on presynaptic responses

Katz and Miledi (1965, 1968) and Braun and Schmidt (1966) demonstrated active impulse conduction along most of the length of frog motor terminals. To get a more precise estimation of the actual length of the excitable portion I ionophoretically applied TTX close to the recording electrode while looking for changes in electrical signals at different electrode emplacements.

The effect of TTX on proximal and distal waveforms is shown in Fig. 4. Proximal responses (electrode approximately at position C of Fig. 1) are diphasic; absence of late positive deflection does not necessarily mean absence of outward K current, as shown above. TTX application (A2) suppressed the sharp negative component that signals regenerative inward Na current (Brigant and Mallart 1982). Early positivity increases in size and is followed by second positive deflection. The most likely explanation is that this part becomes inexcitable and is passively invaded by local circuit current from upstream nodes of Ranvier. Early positivity thus signals a source of current large enough to depolarize the membrane and activate potential dependent K channel which generate a late outward current. The latter was not visible before TTX application because it was masked by a sink of current caused by active K current at more distal parts of the terminal branch. Interestingly, the TTX-modified response recorded from the initial part of frog terminals closely resembles the response recorded under normal conditions from the inexcitable distal part of mouse motor endings (Brigant and Mallart 1982; Mallart and Brigant 1982).

The effect of TTX application to more distal areas is shown in Fig. 4B. The locus corresponds approximately to H in Fig. 1 where the response is a predominantly positive diphasic waveform. TTX induces an increase in amplitude and duration of the positive spike and a reduction of the negative phase. This effect is clearly seen in Fig. 4B3 where traces taken before and during (arrow) drug application are superimposed. This result indicates that, under normal conditions, early outward passive current flow is terminated here by active inward Na current. However, from the relative size of TTX-sensitive and -insensitive components, one can tell that Na current density ought to be lower here compared to that in more proximal portions. TTX application upon points located in the last quarter of the non-myelinated branch failed to induce appreciable changes in the configuration of recorded waveform (Fig. 4C), which indicates a very low density or absence of Na channels.

The apparent low density of Na channels in distal nerve terminals suggests that early passive current at distal parts may be caused by active Na current at more proximal sites. Consistent with this possibility, positive and negative peaks are practically coincident in time. For istance, in the recordings of Fig. 1 differences in peak latency between negative spikes at points E, F, G and H and positive spikes at points J, K and L is only about 50 μs, which is within experimental error. This observation suggests absence of active propagation distal to a point situated roughly half-way between the

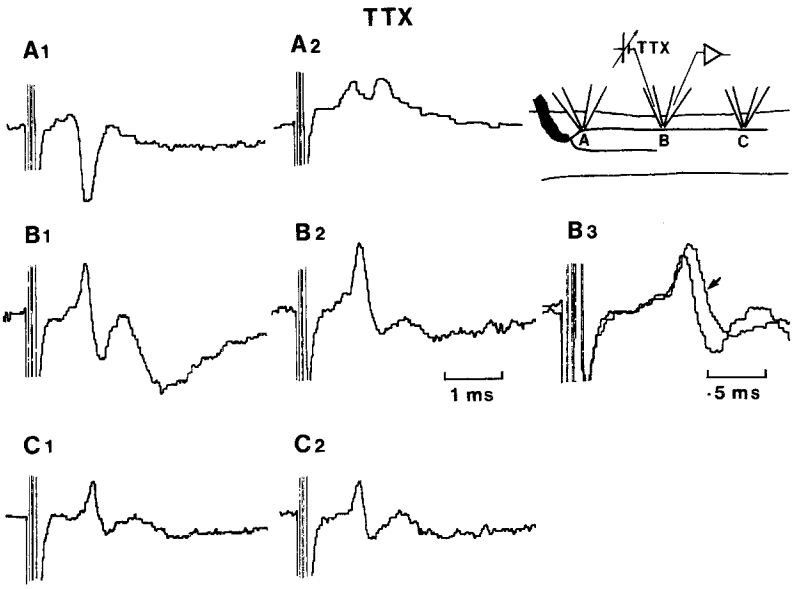

Fig. 4A–C
Effect of ionophoretic TTX on waveform components. **A1**, **B1** and **C1** Control responses from proximal, medial and distal parts, respetively, of motor terminals. Ionophoretic TTX application at a given electrode emplacement modifies proximal and medial, but not distal waveforms. TTX modified proximal responses consist in early passive outward current followed by late outward active K current. Positive spike broadening and near suppression of late negativity of medial responses by TTX is illustrated in **B3** where **B1** and **B2** have been superimposed at a faster time base. Note that end-plate current, visible in **B1** as a broad negative deflection, is greatly reduced during TTX application. Tubocurarine concentration was 5 μM in **A** and 3 μM in **B** and **C**

myelin and the extreme tip. This point would correspond approximately to H-I in Fig. 1 where important changes in the relative importance of positive and negative waveform components occur, yet the signal still retains a triphasic configuration which indicates impulse conduction (see Katz and Miledi 1965). Although experiments similar to that illustrated in Fig. 4B indicate presence of Na channels just distal to this point, it is questionable whether their density is sufficient to sustain full amplitude impulse propagation. Relevant to this matter is the observation of increased conduction velocity at distal portions of frog endings (Braun and Schmidt 1966). Probably, it corresponds to average conduction velocity in portions of non-myelinated branches which include one segment of active conduction and another of passive, instantaneous, propagation.

Potential change at the end of frog motor terminals

If action potentials do not actively invade the distal end of nerve terminals, potential change there would depend on electrotonic attenuation of action potentials generated at proximal parts. Since local circuit current recorded by a focal electrode positioned at distal parts is proportional to potential difference between distal and proximal portions of nerve terminal, it is possible to obtain a rough estimate of potential changes at the former from focal current recordings and action potential configuration in the axon (Eccles 1964, Fig. 46; Brigant and Mallart 1982). Such a reconstruction is shown in Fig. 5 where propagated action potential in a motor axon at 20° C (A, continuous line) is taken from Stämpfli and Hille (1976) and B is focal current recording from the present series of experiments. The inferred potential change at the terminal end is given by the broken line in A.

Calcium current

"Calcium current" may be recorded as negative or positive deflections from distal or proximal parts, respectively, of mouse motor endings within few minutes of perfusion with K channel blockers (Mallart and Brigant 1982). Similar wave-

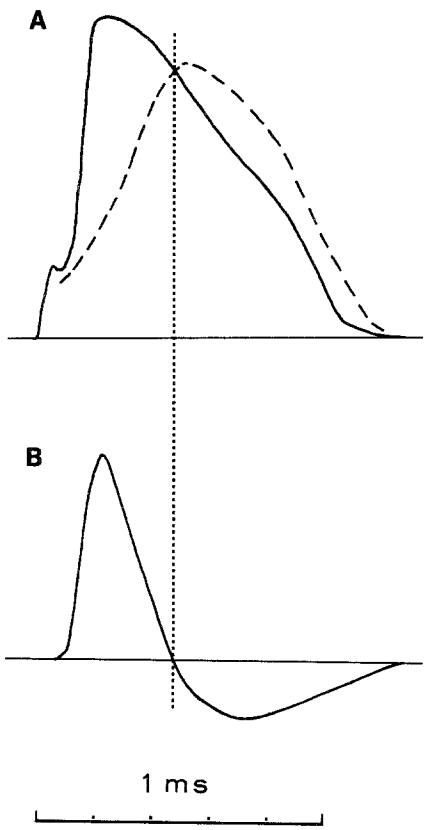

Fig. 5A, B. Reconstruction of potential changes near terminal end. *Solid line* in **A** represents an action potential recorded in a node of Ranvier from a frog motor nerve fibre by Stämpfli and Hille (1976) at 20° C. **B** Focal current recording from the distal part of frog endings at the same temperature. *Dashed line* in **A** represents the reconstructed potential change in distal portions assuming passive invasion. Rise time is prolonged and amplitude is reduced by cable properties; decay time is little affected because some degree of K activation occurs. At distal parts, lower potential during the rising phase and higher potential during the decay generate a source and a sink of current, respectively. *Dotted line* indicates isopotentiality between proximal and distal parts and reversal of membrane current

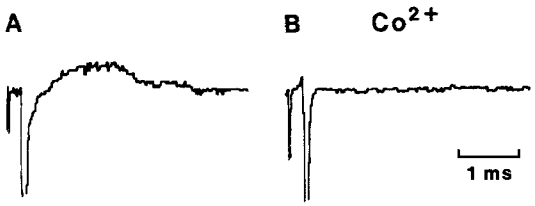

Fig. 6A, B. "Calcium current". 1 mM TEA and 0.1 mM 3,4-DAP present throughout. Recording electrode positioned near the myelin. Outward current in **A** corresponds to inward Ca current at the main part of the endings. **B** Shows abolition of this current by bath perfusion of 5 mM Co^{2+}. Sharp negative spike is truncated in the tracings

forms could be obtained from TEA + 3,4-DAP treated frog endings although their amplitude, relative to the Na dependent deflection, was much lower. The recordings of Fig. 6 were taken at the transition between myelinated and non-myelinated portions where active Ca current entering the membrane at the main part of the endings is seen as outward current (A). The addition of 5 mM Co^{2+} to the bath suppressed it readily (B). One possible explanation for the relatively low amplitude of Ca responses in our experimental conditions is that Ca channels are uniformly distributed along the non-myelinated stretch where each point may act simultaneously as a sink and source of Ca-dependent current. In agreement with this interpretation is the observation of a large outward Co^{2+} sensitive current in frog myelinated preterminal axons where Ca channels are not expected to occur (Gundersen et al. 1982).

Discussion

The results of this study point to a non uniform distribution of Na and K channels along non-myelinated parts of frog motor nerve terminals which lead to local changes in membrane excitability. The evidence derives from regional variations in waveform configuration and actions of specific channel blocking agents on waveform components. Electrical signals recorded from frog endings consist, starting close to the myelin, of a two component negative, a triphasic and, finally a diphasic waveform. These proximo-distal changes in shape cannot be entirely interpreted in terms of currents associated to action potential propagation in a linear, closed end structure endowed with uniform ionic properties. The observed changes in polaritiy of late currents can rather be explained by assuming that the decay phase of the action potential is shorter in portions where late current is outward compared to portions where late current is inward, the former acting as a source and the latter as a sink of local circuit current. Regional variations in K channel density can thus account for late current polaritiy. This view is strongly supported by the effect of local application of K channel blocking agents on amplitude and duration of late components of the recorded signals.

Na channels seem to be virtually absent from the distal part of frog terminals which are, thus, electrically inexcitable. In this connection, Katz and Miledi (1965) failed frequently to elicit antidromic spikes on distal end stimulation. It is uncertain whether Na channel density falls abruptly or starts decaying progressively at some distance before the end. Probably, channel density becomes gradually lower near the middle of the branch as suggested by the relatively moderate effect of TTX applied to medio-distal areas. If this is the case, one would expect propagation over a short distance of lower than normal amplitude action potentials. In an attempt to give a simplified view, one can say that for a 160 μm long branch, action potentials are conducted full size for the first 80 μm, then at lower amplitude for the next 40 μm and, finally by passive spread over the last 40 μm. By assuming a 60 μm space constant (Katz and Miledi 1968), depolarization might not be sufficient to active Ca channel at the very tip. Indeed I have observed 100 % failure of release near the terminal end and only 1 % failure at more proximal segments (unpublished observations in Mg treated preparations). This might also be due to a lack of voltage sensitive Ca channels or release sites at the distal end.

Presynaptic channel distribution in frog contrasts with that found in mouse endings where Na channels are separated from K and Ca channels and from transmitter release sites (Brigant and Mallart 1982). In this respect, the situation in frog terminals resembles that described in crayfish, where release sites occur in both excitable and inexcitable parts of the presynaptic terminal (Zucker 1974; Atwood 1967; Thieffry and Bruner 1978; Dudel 1982).

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